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RESEARCH ARTICLE

Development and optimization of a double antibody sandwich ELISA for the detection of goose T cell surface CD8α molecule

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Abstract

CD8, a glycoprotein on the surface of T cells, is involved in the defense against viral infection and plays significant roles in antigen presentation and in the antiviral immune response. CD8 is composed of two chains. Of these, the CD8α chain was chosen for the detection because it involved in both the CD8αα homodimer and the CD8αβ heterodimer. Here, we established a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for specific detection of goose CD8α (goCD8α). The results showed that the optimal coated antibody and antigen dilutions were 1:50 (the antibody titer was 1:12 800) and 1:32 (0.3 ng mL^{-1}), respectively, while the optimal capture antibody and horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG dilutions were 1:50 (the antibody titer was 1:51 200) and 1:4 000 (the antibody titer was 1:5 000), respectively. The optimal blocking buffer was 5% bovine serum albumin (BSA). The best incubating condition was overnight at 4°C, the best blocking time was 120 min and the best anti-capture antibody working time was 150 min. In addition, the minimum dose detectable by DAS-ELISA was 5×10^{-3} ng mL^{-1}. Most importantly, goCD8α expression levels in goose spleen mononuclear cells (MNCs) post-Goose parvoviruse (GPV) infection were found to be significantly up-regulated using the DAS-ELISA method, which was consistent with previous results obtained using real-time quantitative PCR. In conclusion, the DAS-ELISA method reported here is a novel, specific technique for the clinical detection of goCD8α.

Keywords: T cells, goose CD8α, polyclonal antibody, double antibody sandwich ELISA

1. Introduction

T cells are composed of lymphoid stem cells within the thymus. They are also the key components of lymphocytes, which play an important role in the mutual interaction between hosts and pathogens (Powell *et al.* 2009). According to their immune function, these cells can be divided into several subsets with distinct function (Erf 2004). Importantly, CD8 T cells, are also known as cytotoxic T cells (CTLs) with CD8

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glycoprotein expressed on their surfaces. As non-polymorphic molecules with cell surface glycoproteins, CD8 molecules are widely expressed on CTLs and also function as co-receptors for the polymorphic T cell receptor (TCR) in antigen recognition by binding to the constant region of major histocompatibility complex (MHC) class I proteins (Luhtala 1997, 1998). CD8 T cells also serve to irreparably damage pathogen-infected cells by secreting perforin and granzymes from endocellular vesicles or by inducing apoptosis through Fas receptor activation (Kägi *et al.* 1994; Michele and Chris 2002). In addition, CD8+ T cells mainly produce cytokines such as interferon-γ (IFN-γ) and tumornecrosisfactor (TNF)-α, which further activate macrophages and inhibit viral replication (Chen *et al.* 2015). In summary, CD8 is a marker of CD8+ T cells, which are expressed on the surface of cells and are involved in the clearance of viruses, antigen presentation and the immune response (Zhao *et al.* 2013).

The CD8 molecule has two isomers, αβ heterodimers and αα homodimers, which have different biochemical structures and tissue distribution profiles (DiSanto *et al.* 1988; Norment and Littman 1988). Considering that both CD8αα and CD8αβ contain the CD8α chain, CD8α was chosen for the detection of CD8 molecules in this study. A double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) has previously been developed for the detection of human CD8 molecules (Carriere *et al.* 1994) and is operational in distinguishing between healthy subjects and viral-infected patients. Recently, ELISA kits have emerged for using the recognition of goose CD8 (MyBioSource, Cat. No. MBS010466 & Cat. No. MBS036368). However, the CD8 antibody used in these ELISA kits was based on human CD8 or chicken CD8, not goose CD8. Based on the goose cDNA sequence that we previously characterized (Zhao *et al.* 2013), we developed a method for the specific detection of goose CD8α. Two polyclonal antibodies (PAbs) were obtained based on goose CD8α (goCD8α), and in light of this, we established a DAS-ELISA to specifically detect goCD8α. Our study provides a specific detection method for goose CD8α expression level, which can be used in clinical detection and experimental research.

2. Materials and methods

2.1. Establishment and optimization of DAS-ELISA

Initially, the extracellular region without the goCD8α signal peptide gene was amplified with the specific primers and then cloned into a pET-32a(+) vector to obtain goCD8α-exc-pET-32a. To establish an efficient DAS-ELISA method, the recombinant protein (goCD8α-exc) (Cheng *et al.* 2016) was purified and used as the detective antigen during the DAS-ELISA. The titer of the coated antibody (mouse anti-his-goCD8α-exc PAb) was 1:12 800 (Cheng *et al.* 2016) and was diluted in a bicarbonate buffer (0.05 mol L^{-1}, pH=9.6) before being used to coat 96-well microplates (100 μL per well). In the same way, non-immunogenic serum was used as the negative control. After incubation, the wells were washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) and then blocked. Subsequently, the recombinant protein (goCD8α-exc) was added and incubated. Then, the antibody titer of the capture antibody (rabbit anti-his-goCD8α-exc PAb, 1:51 200) (Cheng *et al.* 2016) was added to each well after three washes and was incubated. Thereafter, the wells were washed three times with PBST and incubated with 100 μL of horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (Sangon Biotech, Shanghai). Finally, wells were detected using an EL-TMB Chromogenic Reagent Kit (Sangon Biotech, Shanghai). Additionally, the OD_{450} values of each well were read by using an iMark microplate reader (Bio-Rad, Japan).

Based on the best antigen concentration, the optimal concentration of detection antibody was determined through standard checkerboard titration procedures. The coated antibody was immobilized into 96-well microplates at different dilutions ranging from 1:50 to 1:1 600 (100 μg per well), and non-immunogenic sera of the same dilution gradient were used as negative controls. After washing with PBST for three times, the recombinant protein was added in a serial dilutions from 1:2 to 1:256. A phalanx titration was used to determine the best working conditions. After establishing the antigen concentration and detection antibody dilution gradient, working concentrations of capture antibody and HRP-labelled goat anti-rabbit IgG were optimized using standard checkerboard titration procedures. The dilutions of the capture antibody ranged from 1:50 to 1:6400, and the dilutions of the anti-capture antibody ranged from 1:500 to 1:5000. According to the previous research, the highest P/N value (>2.1) and the OD_{450} value of positive serum closest to 1.0 were selected as optimal working conditions.

Additional optimizations were performed based on the antibody dilution procedures, including those for best incubation conditions, blocking buffer, blocking time and anti-capture antibody working time (Table 1).

2.2. Detection range of DAS-ELISA

Under the determined best conditions, the different concentrations (5×10^{-1}, 2.5×10^{-1}, 1.25×10^{-1}, 1×10^{-1}, 5×10^{-2}, 2.5×10^{-2}, 1.25×10^{-2} and 5×10^{-3} ng mL^{-1}) of goCD8α-exc were detected by the established DAS-ELISA.

2.3. Detection of GPV-infected MNCs by DAS-ELISA

For demonstrating the availability of the established

DAS-ELISA method, the goose spleen mononuclear cells (MNCs) were chosen as models cells and a Goose parvovirus (GPV) strain (kept by the Avian Diseases Research Centre of Sichuan Agricultural University, China) was chosen as the stimulators. The median egg infectious dose (EID_{50}) of the GPV strain suspension was $10^{-6.6}$ 0.2 mL^{-1} (Chen *et al.* 2005; Yang *et al.* 2009), and the expression level of goCD8α was detected by using the established DAS-ELISA. Briefly, the MNCs were prepared according to the literature (Chen *et al.* 2015), and the cells were stimulated with GPV (infected group; 25 μL, containing 10 EID_{50}), while the controls were treated with PBS (mock-infected group; 25 μL). At 72 h post infection, the cells were washed three times and detected using established DAS-ELISA. The OD_{450} value was recorded using an iMark Microplate Reader (Bio-Rad, Japan)

3. Results

3.1. DAS-ELISA development and optimization

The highest P/N (i.e., OD_{450} value of positive serum/OD_{450} value of negative serum) value (>2.1) was used to optimize the experimental conditions. The results (Table 2) showed that the best working dilutions of the coated antibody and antigen were 1:50 (the antibody titer was 1:12 800) and 1:32 (0.3 ng mL^{-1}), respectively, with an OD_{450} of 1.24. Subsequently, the working concentrations of the detected antibody and HRP-labelled goat anti-rabbit IgG were determined (Table 3) as 1:50 (the antibody titer was 1:51 200) and 1:4 000 (the antibody titer was 1:5 000), respectively. Then, optimization of other conditions was carried out. The results (Fig. 1) showed that the best incubating condition was overnight at 4°C, the best blocking buffer was 5% bovine serum albumin (BSA), the best blocking time was 120 min, and the best anti-capture antibody working time was 150 min.

3.2. Detection limits of DAS-ELISA

The highest P/N value (>2.1) and the OD_{450} value of positive serum closest to 1.0 were selected as optimal working conditions. The results (Fig. 2) showed that the minimum detectable dose of DAS-ELISA for goCD8α-exc protein was 5×10^{-3} ng mL^{-1}.

Table 1 The optimization of the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for goose CD8α (goCD8α)

Optimization	Options
Incubating condition	4°C overnight; 4°C for 16 h; 37°C for 2 h; 37°C for 4 h; 37°C for 8 h; 37°C overnight
Blocking buffer	1% skim milk; 3% skim milk; 5% skim milk; 1% BSA; 3% BSA; 5% BSA
Blocking time	30 min; 45 min; 60 min; 120 min; 150 min
Incubating time (anti-capture antibody)	30 min; 45 min; 60 min; 120 min; 150 min

Table 2 The optimization on the dilution of coated antibody and antigen (goCD8α-exc)

Dilution of coated antibody	Dilution of antigen (goCD8α-exc)								
	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	OD_{450} value[1)]
1:50	1.220	1.250	1.220	1.200	1.240	1.280	1.310	1.300	P
	0.299	0.301	0.315	0.312	0.272	0.298	0.314	0.321	N
	4.080	4.150	3.870	3.850	4.560	4.300	4.170	4.050	P/N
1:100	1.160	1.160	1.190	1.180	1.100	1.220	1.200	1.270	P
	0.265	0.264	0.274	0.264	0.285	0.271	0.278	0.285	N
	4.380	4.390	4.340	4.470	3.860	4.500	4.320	4.460	P/N
1:200	1.040	1.010	1.000	0.980	1.010	1.030	1.020	1.022	P
	0.245	0.248	0.253	0.246	0.257	0.256	0.250	0.247	N
	4.240	4.070	3.950	3.980	3.930	4.020	4.080	4.140	P/N
1:400	0.998	1.000	1.000	0.936	0.870	0.84	0.790	0.756	P
	0.269	0.259	0.255	0.249	0.229	0.241	0.221	0.213	N
	3.710	3.860	3.920	3.760	3.800	3.490	3.570	3.550	P/N
1:800	0.570	0.680	0.690	0.590	0.550	0.542	0.300	0.490	P
	0.290	0.256	0.271	0.241	0.232	0.235	0.213	0.229	N
	1.970	2.660	2.550	2.45	2.370	2.310	1.410	2.140	P/N
1:1 600	0.308	0.306	0.302	0.29	0.262	0.246	0.240	0.245	P
	0.247	0.228	0.232	0.216	0.204	0.194	0.187	0.189	N
	1.250	1.340	1.300	1.340	1.280	1.270	1.280	1.300	P/N

[1)] P, OD_{450} value of positive serum; N, OD_{450} value of negative serum; P/N, OD_{450} value of positive serum/negative serum. The same as below.

Table 3 The optimization on the dilution of capture antibody and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG

Dilution of capture antibody	Dilution of HRP-labeled goat anti-rabbit IgG						
	1:500	1:1 000	1:2 000	1:3 000	1:4 000	1:5 000	OD_{450} value
1:50	1.324	1.305	1.264	1.204	1.121	1.040	P
	0.310	0.296	0.290	0.272	0.241	0.235	N
	4.277	4.408	4.364	4.426	4.647	4.420	P/N
1:100	1.258	1.209	1.190	1.079	0.985	0.971	P
	0.313	0.291	0.294	0.278	0.253	0.240	N
	4.016	4.155	4.041	3.886	3.900	4.002	P/N
1:200	0.933	0.698	0.894	0.808	0.730	0.722	P
	0.255	0.259	0.255	0.229	0.215	0.195	N
	3.657	2.693	3.504	3.531	3.398	3.694	P/N
1:400	0.760	0.756	0.776	0.725	0.723	0.720	P
	0.223	0.221	0.224	0.211	0.203	0.203	N
	3.410	3.427	3.469	3.438	3.569	3.554	P/N
1:800	0.692	0.654	0.593	0.606	0.580	0.547	P
	0.218	0.224	0.200	0.206	0.183	0.187	N
	3.174	2.920	2.965	2.942	3.169	2.925	P/N
1:1 600	0.523	0.511	0.500	0.488	0.492	0.482	P
	0.208	0.207	0.201	0.195	0.189	0.189	N
	2.510	2.473	2.482	2.497	2.604	2.548	P/N
1:3 200	0.311	0.309	0.301	0.283	0.290	0.279	P
	0.128	0.128	0.124	0.122	0.117	0.119	N
	2.427	2.418	2.428	2.324	2.485	2.343	P/N
1:6 400	0.272	0.259	0.233	0.207	0.207	0.200	P
	0.123	0.126	0.137	0.110	0.115	0.108	N
	2.211	2.056	1.701	1.882	1.800	1.852	P/N

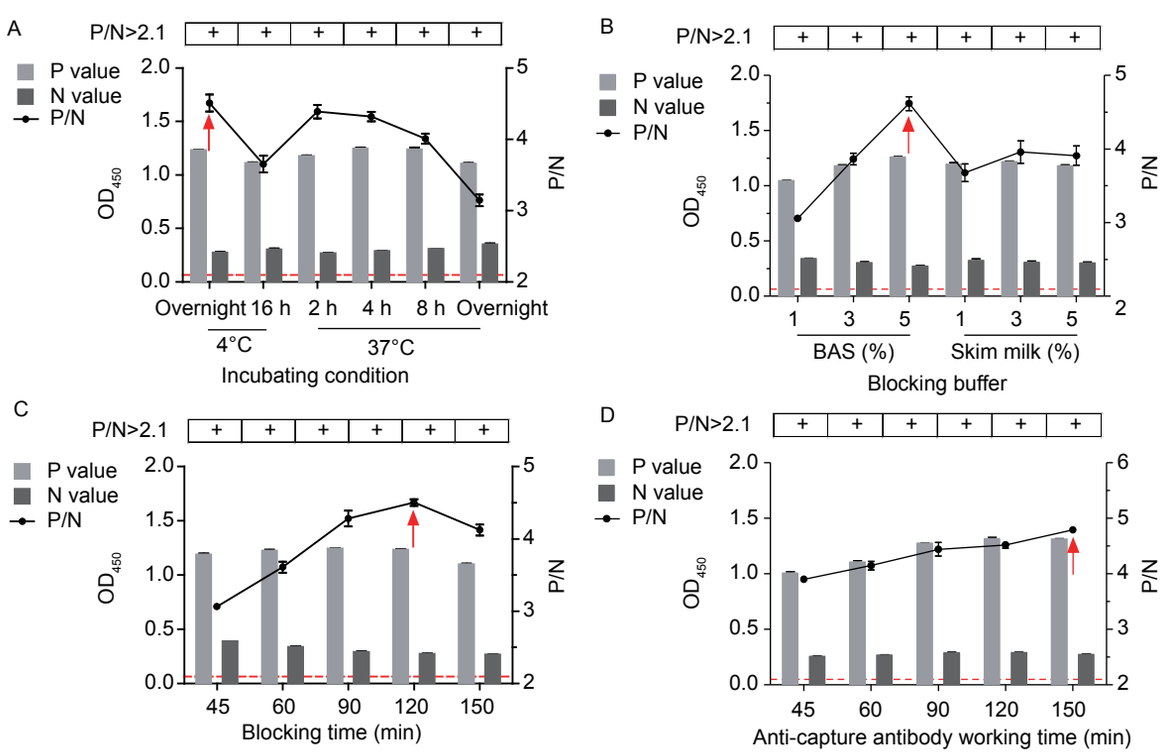

Fig. 1 Optimization condition of a double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). A, selection of incubating condition. B, selection of blocking buffer. C, selection of blocking time. D, selection of the anti-capture antibody working time. The P/N (i.e., OD_{450} value of positive serum/OD_{450} value of negative serum) value of each group was showed on the each bar (right *Y* axis), P/N value (>2.1) was determined to be positive results (+). The best condition (or the highest P/N value) of each other was pointed by red arrows (↑).

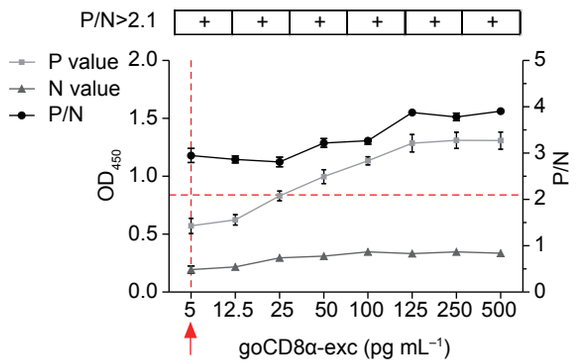

Fig. 2 The minimum detectable of antigen goCD8α-exc by DAS-ELSIA. Different numbers of cells were seeded in 96-well plate respectively, and three replicates of each were performed. The positive serum diluted in PBS was added in the positive group, and the non-immunogenic sera were used as negative control. The P/N value of each group was showed on the each bar (right *Y* axis), P/N value (>2.1) was determined to be positive results (+). The minimum detectable of goCD8α-exc was pointed by the red arrow (↑).

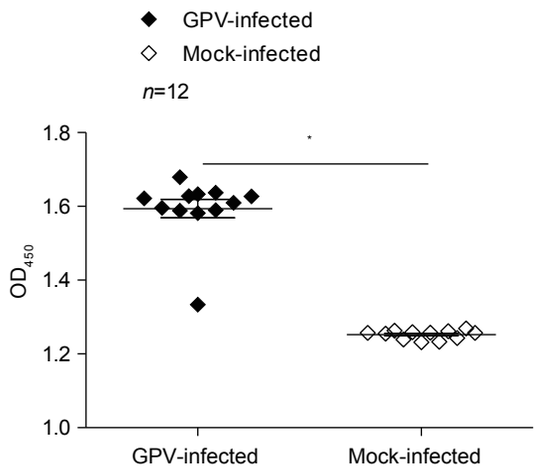

Fig. 3 Detection of goCD8α in the Goose parvoviruse (GPV)-infected and mock-infected mononuclear cells (MNCs) by DAS-ELISA. Three groups of cells treated with 25 μL GPV suspension (containing 10 EID_{50}) were served as the infected group, and the mock-infected groups were treated with 25 μL PBS as the control. Then the expression of CD8α molecular was detected at 72 h after infection, twelve replicates of each group were performed, and the OD_{450} value was recorded, significant difference at $P<0.05$ was pointed by *.

3.3. Detection of GPV-infected MNCs by DAS-ELISA

The highest P/N value (>2.1) and the OD_{450} value of positive serum closest to 1.0 were selected as optimal working conditions. The results (Fig. 3) showed that the OD_{450} value of the GPV-infected MNCs was higher than that of the mock-infected cells, and the difference between the two groups was significant ($P<0.05$).

4. Discussion

CD8α is a useful marker for cellular immune response and for the development of some immunological kits. Previously, CD8α antibody has been reported in duck (Kothlow *et al.* 2005), chicken (Luhtala 1995), and turkey (Powell *et al.* 2009). In our previous studies (Cheng *et al.* 2016), we used goose CD8α to develop a cellular ELISA (2.5×10^4 cells per 100 μL) for the detection of goose CD8α, but it cannot rapidly detect goose CD8α in clinical diagnosis. Therefore, we established a method for the specific and rapid detection of goCD8α. In addition, the transcription levels of goCD8α mRNA were detected *via* real-time quantitative reverse transcription PCR (qRT-PCR) (Zhao *et al.* 2014) and the development and tissue distribution of CD8α in goose embryo, gosling and goose were determined *via* immunocytochemistry using anti-duck CD4/CD8α monoclonal antibodies (Chen *et al.* 2015). However, qRT-PCR only reveals the mRNA level of goCD8α, requires equipment, and is time-consuming. Additionally, immunocytochemistry cannot be used to determine the percentage of CD8α positive T cells. Thus, it is evident that establishing a novel, easily performed and convenient assay is required for clinical practice, especially for the routine detection of goCD8α. Among the many methods available for rapid detection of viral infections, DAS-ELISA is the best way to perform mass screening clinical specimens. In this experiment, a DAS-ELISA method, based on two PAbs against the recombinant protein goCD8α-exc, with mouse anti-his-goCD8α-exc PAb serving as the coated antibody, and rabbit anti-his-goCD8α-exc PAb serving as the capture antibody. We also attempted to use rabbit anti-his-goCD8α-exc PAb as a capture antibody in the development of this assay; however, the results showed that rabbit anti-his-goCD8α-exc PAb was unsuitable for detection when rabbit anti-his-goCD8α-exc PAb was used as the coated antibody, because of the high negative OD_{450} value and low positive OD_{450} values. Based on this, we chose the mouse anti-his-goCD8α-exc PAbs and rabbit anti-his-goCD8α-exc PAbs to act as the coated and capture antibodies, respectively. Subsequently, the assay was successfully established based on two types of anti-his-goCD8α-exc Pabs.

As seen from the results, the minimum dose detectable by DAS-ELISA was 5×10^{-3} ng mL^{-1}. This suggests the potential for clinical use of the established method, which can detect goose CD8α with high sensitivity. Additionally, detection of GPV-infected MNCs by the DAS-ELISA method showed that the infected group has significantly up-regulated relative to uninfected group, which is consistent with our previous report (Zhao *et al.* 2014), demonstrating that the

established DAS-ELISA is a suitable and specific method for detecting goCD8α.

5. Conclusion

The DAS-ELISA technique is a novel, convenient and highly useful method for the detection. We also confirmed that DAS-ELISA might be of great significance in further understanding of goose immunological research.

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